

Molecular Identification of Entomopathogenic Nematode Isolates from the Philippines and their Biological Control Potential Against Lepidopteran Pests of Corn

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Abstract

In search for local entomopathogenic nematode (EPN) species as a biological control agent of lepidopterous insect pests of corn, a survey for EPN in the major islands in the Philippines was conducted. Seven EPN populations from 279 soil samples were isolated using *Ostrinia furnacalis*, the key target insect pest of corn in the country, as bait. Analysis of the ITS1-5.8S-ITS2 ribosomal DNA sequence revealed the presence of *Steinernema abbasi*, *Steinernema minutum*, *Steinernema tami*, and *Heterorhabditis indica*. The pathogenicity of these EPN was tested in *Ostrinia furnacalis*, *Spodoptera litura*, and *Helicoverpa armigera* larvae under laboratory conditions. All the EPN isolates were pathogenic to the lepidopteran species with, *H. indica* PBCB and *S. abbasi* MBLB exhibiting the highest virulence (88%–99.33% and 90%–100% mortality, respectively) at 48 hr post infection (HPI) and thus, further studies were done on these two EPN. The highest penetration rate at 48 HPI was observed in *H. armigera* infected with *S. abbasi* MBLB (28.15%), while the lowest was in *O. furnacalis* infected with *H. indica* PBCB (14.25%). Nonetheless, based on LC₅₀ at 48 HPI, *H. indica* PBCB was most virulent to *S. litura* (8.89 IJ per larva), but not significantly different from *O. furnacalis* (10.52 IJ per larva). *Steinernema abbasi* MBLB was most virulent to *O. furnacalis* (10.98 IJ per larva), but not significantly different to *S. litura* (17.08 IJ per larva). LT₅₀ estimates showed that *O. furnacalis* was significantly the most susceptible to *H. indica* PBCB (21.90 hr) and *S. abbasi* (21.18 hr). Our results suggest that *H. indica* PBCB and *S. abbasi* MBLB are good candidates as biological control agents against these insect pests of corn. Moreover, *O. furnacalis* as alternative bait for EPN was discussed. To date, this is the most extensive research on Philippine EPN, comprised of wide sampling coverage, molecular identification and bioefficacy assays.

Key words

Biological control, Entomopathogenic nematode, *Helicoverpa armigera*, *Heterorhabditis indica*, Internal transcribed spacer, *Ostrinia furnacalis*, Ribosomal DNA region, *Spodoptera litura*, *Steinernema abbasi*.

Entomopathogenic nematodes (EPN) have long been studied for their pronounced virulence against a wide range of insects belonging to orders Lepidoptera, Coleoptera, Diptera, Thysanoptera, and Orthoptera. Their parasitic life cycle is initiated by the infective

juveniles (IJ), either through actively searching (cruisers) or waiting (ambushers) for their insect host. They may enter the host through body openings or by penetrating directly the cuticle to reach the hemocoel. Once in the hemocoel, they release their bacterial endosymbionts

(*Xenorhabdus* and *Photorhabdus* for *Steinernema* and *Heterorhabditis*, respectively) that multiply very rapidly in the hemolymph resulting to host death within 24 to 72 hr. The nematodes then feed on the bacteria and complete their life cycle inside the insect cadavers. They emerge from the deteriorating cadaver, carrying the bacterial symbiont in the anterior part of their intestine to begin another infection cycle (Smart, 1995; Burnell and Stock, 2000).

EPN-based technology provides a biological control option on many of the most important pests of agricultural crops. In addition, EPN can be integrated with other management strategies and reduce growers' dependence on chemical insecticides. The efficacy of EPN has been demonstrated against several insect pests in different countries such as black vine weevil on cranberries (Shanks and Agudelo-Silva, 1990), leaf miner on ornamentals and vegetables (Hara et al., 1993), citrus root weevil (Bullock et al., 1999), mole crickets on turf grass (Barbara and Buss, 2005), and peachtree borer (Shapiro-Ilan et al., 2009) in the United States; European corn borer (Ben-Yakir et al., 1998) in Israel; fall army worm (Negrisoli et al., 2010) in Brazil; western flower thrips in Germany (Ebssa et al., 2001); and Japanese pine sawyer in Asia (Phan, 2008). As a matter of fact, EPN-based commercial products are already available in Europe, the United States, Australia, and in parts of Asia (Georgis and Hom, 1992; Kaya et al., 2006).

EPN are naturally found in both agriculturally disturbed and undisturbed soil environments with reports of occurrences from many temperate and tropical countries. To date, there are at least 90 *Steinernema* and 20 *Heterorhabditis* species reported (Shapiro-Ilan et al., 2017). A number of new additions to this growing list are newly described species from Asia, indicating a high diversity of EPN in the region. Gapasin et al. (2016), although reported the occurrence of EPN in the Philippines, the identification was only at the genus level. In addition, their collection was limited to *Steinernema* spp. and *Heterorhabditis* spp. in sweet potato growing areas in the country.

Corn is one of the most economically important commodity in the Philippines. From 2010 to 2014, the area planted to corn has increased up to 2.6 Mha with a production of ~7.8 Mt (Food and Agriculture Organization of the United Nations, 2017). Many of the Filipino farmers depend on tilling their land for corn production either for feed or food. However, corn production areas have been threatened by the prevalence of different pests and pathogens with the vast majority comprised of herbivorous lepidopterous insects including the Asian corn borer, *Ostrinia*

furnacalis Guenée, which is considered the most serious biotic constraint in corn fields in the country (Camarao, 1983; Gerpacio et al., 2004; Litsinger et al., 2007; Afidchao et al., 2013), as well as the corn earworm, *Helicoverpa armigera* (Hubner), and the common cutworm, *Spodoptera litura* Fabricius (Gerpacio et al., 2004).

Two generations of *O. furnacalis* are observed in a corn cropping season in the Philippines, with the first and second generation coinciding with the vegetative and pollen shedding, respectively. The early stage larvae feed on the leaves and tassel. Camarao (1976) noted that although the late 4th instar larvae tunnel into the stalks and feed until pupation, some remained in the whorled leaves and unopened tassel and spikelets until pupation. *Helicoverpa armigera* and *S. litura* also attack the leaves during the vegetative stage. During reproductive stage however, *H. armigera* prefers to feed on the tassel and cobs (Ramos and Morallo-Rejesus, 1981). Both lepidopterans exhibit overlapping generations in a corn cropping season. The last instar larvae of *H. armigera* migrate toward the soil where they pupate (Smith-Pardo, 2014). The larvae of *S. litura* on the other hand, prefer moist sites and often hide in the soil during the day attacking young corn plants at night. The common cutworm also pupates in the soil (Nurhidayat, 2003).

With the concerns regarding the management and control of these pests, many of the farmers depend on the use of chemical pesticides and deployment of genetically modified corn varieties. However, key issues on the deleterious effects of heavy pesticide usage (Mohankumar and Ramasubramanian, 2014) and the apparent emergence of resistant pest populations (Gassmann et al., 2011; Tabashnik et al., 2013; Tabashnik, 2015) prompted the search for a more sustainable and environmentally-sound technology to address pest problems. As many countries have proven the efficacy of EPN as biological control agents, the development of such technology in the Philippines can be tapped to formulate a new pest management strategy.

With very limited studies on EPN in the country, the study was designed to determine the presence of indigenous EPN species virulent to lepidopterous insect pests of corn using a target insect pest as bait specifically, *O. furnacalis*. The use of *O. furnacalis* as host bait was explored basically because it has the closest phylogenetic relationship with *Galleria mellonella* (Linn.) (Regier et al., 2012) and it is the most damaging among the lepidopteran pests of corn. The study also aimed to investigate EPN distribution, and their biological control potential in order to develop

local EPN populations into a viable technology for insect management in corn-producing areas.

Materials and methods

Soil sample collection and EPN baiting

Soil sampling was done by initially clearing off the soil surface from litter and vegetation and digging a pit to a depth of about 10-cm using a shovel. From that pit, a soil sample weighing ~1.5 kg was collected, placed inside a resealable plastic bag and labelled accordingly. From each site, two to three samples were taken depending on the size of the area. Sampling was conducted in agricultural and forested areas in Albay, Batangas, Camarines Sur, Bukidnon, Cavite, Cebu, Isabela, Laguna, Misamis Oriental, Pangasinan, Pampanga, Quezon, and South Cotabato. Soil samples positive for EPN were analyzed for pH, organic matter, sand, silt, and clay contents. The baiting technique for nematode extraction from soil was carried out by burying five live *O. furnacalis* larvae underneath the moist soil in an aerated 280 cm³ plastic container. Dry soil samples were moistened with distilled water prior to use. Two replicates were prepared for each sample. The setups were incubated in the dark for a week with daily observations to check the insect condition. Dead insects were collected and placed in modified White traps (Kaya and Stock, 1997) or incubated for 3 to 5 days in 60-mm petri plates lined with moistened filter paper for nematode harvesting.

Insect rearing and EPN maintenance

The EPN were mass-reared in *O. furnacalis* larvae. The IJ were stored at ~2,500 IJ per ml in 25 cm² polystyrene tissue culture flasks (Falcon™, Corning, Inc., New York, NY) at room temperature (28.0 ± 2.0°C). For the bioassay experiments *O. furnacalis* (12-day old), *S. litura* (9-day old), and *H. armigera* (9-day old) larvae were used as test insects. The insects were reared in the laboratory using an artificial diet developed by Ceballo and Morallo-Rejesus (1983).

Morphological and molecular identification of EPN

Permanent mounts of first generation female, male, and IJs were prepared for light microscopy examination. Preliminary identification of the EPN up to the genus level was based on general morphology, as described by Nguyen and Hunt (2007) and Nguyen

et al. (2007). Since the local EPN were reared in *O. furnacalis* instead of *G. mellonella*, which is the model host for EPN where morphometric data were based upon, molecular characterization using the internal transcribed spacer 1-5.8S-internal transcribed spacer 2 (ITS) region of the ribosomal DNA was carried out to identify up to species level. The total DNA was extracted from a single first-generation female using a technique slightly modified from Phan et al. (2005). Briefly, each EPN was macerated in 10 µl of worm lysis buffer and transferred into a microcentrifuge tube, to which 5 µl of sterile distilled water and 1 µl proteinase K (600 µg/ml) were added. The samples were then frozen at -20°C in an absolute ethanol bath for an hour followed by incubation at 65°C for another hour and finally at 95°C for 10 min. The tube was centrifuged at 13,000g for a minute and the DNA suspension was used for polymerase chain reaction (PCR).

The ITS region of the local EPN was amplified using the general primers TW81 and AB28 (Joyce et al., 1994). The PCR profile of Nguyen et al. (2004) was followed using a 25 µl PCR reaction mixture composed of 2.5 µl of the DNA suspension, 12.5 µl 2X Taq Polymerase Mix (Vivantis Technologies, Selangor Darul Ehsan, Malaysia), 0.75 µl of 50 mM MgCl₂ (Vivantis Technologies, Selangor Darul Ehsan, Malaysia), 1.0 µl each of forward and reverse primers (10 pM) and 7.25 µl nuclease-free water. The amplicons were checked in 1% agarose gel in 0.5 TBE buffer and then stained with GelRed (www.biotium.com). The amplified DNA fragments were sent to Beijing Genomics Institute-Hongkong (www.bgi.com) for sequencing. The nucleotide sequences obtained were aligned and edited using BioEdit Version 7.1.10 (Hall, 1999). The deduced consensus sequence for each EPN isolate was compared with the nucleotide sequences in NCBI database using BLASTn (Altschul et al., 1990).

Pathogenicity assay

The pathogenicity tests were carried in 35-mm petri plates (BD Falcon) lined with Whatman™ filter paper No.1. Each plate, representing a replicate, was inoculated with 250 µl of the EPN suspension in distilled water containing 1,000 IJ. Five larvae of each of the test insect species namely, *O. furnacalis*, *H. armigera*, and *S. litura*, were transferred into a petri plate and considered as a replicate. For each insect species tested, ten replicates were used for each EPN strain per trial. Each trial included a control group treated with sterile distilled water. Three repetitions of the trial were done. The plates were then incubated at room

temperature ($28^{\circ}\text{C} \pm 2.0^{\circ}\text{C}$) under dark conditions. Mortality and penetration rates were scored at 48 and 72 hr post infection (HPI), respectively. Two cadavers were dissected to confirm EPN penetration, while the remaining ones were kept until IJ emergence. The harvested nematodes from these test insects were re-inoculated to further confirm pathogenicity to the test insects.

Virulence and penetration bioassays

Virulence, as indicated by mortality, and penetration rates were done for *O. furnacalis*, *H. armigera*, and *S. litura* in 35-mm diameter petri plates lined with filter paper. Each plate was inoculated with 200 IJ in 100 μl of distilled water. A single larva of each test insect species, initially surface-sterilized using 10% hydrogen peroxide and then rinsed thrice in sterile distilled water, was transferred into each plate. The plates were incubated at $28^{\circ}\text{C} \pm 2.0^{\circ}\text{C}$ for 48 hr. Adult EPN were observed and counted. Insect mortality and penetration were expressed as percentage. Five replicates per EPN isolate, with ten larvae per replicate, were used ($n = 50$ larvae) for each insect species in a trial. A control group treated with distilled water was included in the trial. Three repetitions of the trials were done for each test insect. Fresh IJ were used for each trial. Two-way ANOVA analysis was carried out using Prism 6.0 (GraphPad Software, Inc., www.graphpad.com).

Median lethal concentration (LC_{50}) and median lethal time (LT_{50}) estimation:

Bioassays for LC_{50} estimation were carried out in sterile 24-well cell culture plates lined with sterile Whatman No.1 filter paper. A larva was transferred into each well before IJ inoculation. The LC_{50} of *S. abbasi* and *H. indica* on *O. furnacalis* larvae was estimated using the concentrations 2, 4, 8, 16, and 32 IJ per larva, while 4, 8, 16, 32, and 64 IJ per larva were used for *H. armigera*, and *S. litura*. The concentrations used for the bioassays were based on the results of the penetration assays.

Meanwhile, LT_{50} bioassays were carried out in sterile 35-mm diameter petri plates lined with sterile filter paper inoculated with 150 μl nematode suspension with 200 IJ. A larva was placed per plate and sealed with parafilm. Mortality was observed at 2-hour intervals in insects inoculated with *H. indica*, while every 4 hr in insects inoculated with *S. abbasi*. Mortality was recorded up to 48 HPI. LC and LT values were calculated from three trials. Each trial used 30 larvae per EPN species. A control group treated

with sterile distilled water was included in each trial. The LC_{50} and LT_{50} were estimated using Polo Plus 2.0 software (LeOra Software LLC). Differences among LC_{50} or LT_{50} values were considered significant when the 95% fiducial limits did not overlap.

Results

Species identification of the local EPN populations

Steinernema spp. were detected from soil samples from Batangas, Bukidnon, Laguna, and South Cotabato, while the lone *Heterorhabditis* sp. was found from Batangas. Nucleotide sequence analysis of the ITS region further confirmed the presence of these EPN in the soil samples. The ITS region of the local EPNs shared 98% to 100% nucleotide identity with the nucleotide sequences in GenBank database (www.ncbi.nlm.gov/genbank/), indicating the existence of four species from the seven positive soil samples comprised of *Steinernema tami* (1), *Steinernema minutum* (4), *S. abbasi* (1), and *H. indica* (1) (Fig. 1 and Table 1).

Pathogenicity and virulence tests

Preliminary bioassays studies on the seven EPN isolates revealed their pathogenicity against *H. armigera*, *O. furnacalis*, and *S. litura* larvae. All EPN isolates infected and completed their life cycle in all of these lepidopterous insects, however, their virulence, based on larval mortality varied among the EPN isolates and insect species. Results showed that *H. indica* PBCB and *S. abbasi* MBLB, were significantly highly virulent to all the lepidopteran species tested as compared to the *S. minutum* and *S. tami* isolates. As early as 24 HPI, *H. indica* PBCB and *S. abbasi* MBLB caused significantly higher mortality rate in all the test insects ($P < 0.0001$; $\alpha = 0.05$), a trend that continued until 48 HPI (Fig. 2). At 48 HPI, a 99.33% and 100% mortality in *O. furnacalis* was observed for *H. indica* PBCB and *S. abbasi* MBLB, respectively, while a highly significantly lower mortality ($P < 0.0001$, $\alpha = 0.05$), was observed for *S. minutum* ASPL3 (8.0%), *S. minutum* CCL4 (2.0%), *S. minutum* PIB2 (8.0%), *S. minutum* SRSTB3 (16.0%) and *S. tami* PKSC1 (6.67%). Similarly, *H. indica* PBCB and *S. abbasi* MBLB caused significantly higher mortality in both *H. armigera* (88.0% and 90.0%) and *S. litura* (88.67% and 92.00%). Thus, since the local *S. minutum* and *S. tami* isolates exhibited relatively very low mortality on the three corn insect pests tested, only *H. indica* PBCB and *S. abbasi* MBLB were assayed in the succeeding studies.

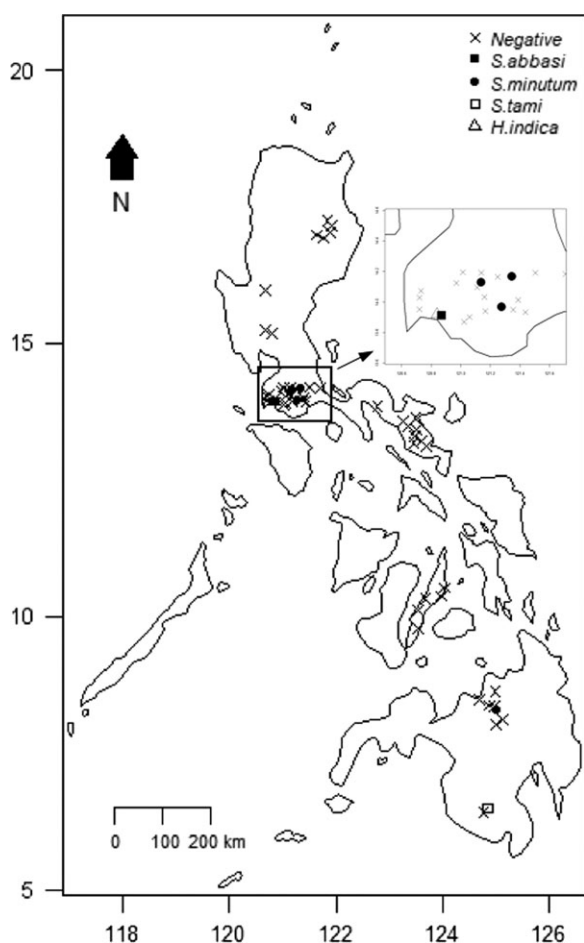


Figure 1: The Philippine map showing the sampling sites for entomopathogenic nematodes. The occurrence and distribution of *Heterorhabditis indica* (Δ), *Steinernema abbasi* (\blacksquare), *Steinernema minutum* (\bullet), and *Steinernema tami* (\square). Collection sites where no entomopathogenic nematodes (\times) was isolated were also indicated. The figure was created using “maps” (Minka and Deckmyn, 2017) and “GIS tools” (Brunsdon and Chen, 2014) libraries in R software.

Percentage IJ penetration

The percentage IJ penetration at 48 HPI of *H. indica* PBCB and *S. abbasi* MBLB in the three lepidopteran species is summarized in Table 2. The penetration rate of *S. abbasi* MBLB in *H. armigera* (28.15%) and *S. litura* (23.15%) was significantly higher than *H. indica* PBCB (14.35% and 18.18%) ($P < 0.0001$, $\alpha = 0.05$). In *O. furnacalis*, however, no significant difference in

the penetration rate of *H. indica* PBCB (14.25%) and *S. abbasi* (14.54%) was observed.

Lethal concentration estimation

The median lethal concentrations (LC_{50}) of *H. indica* PBCB and *S. abbasi* MBLB at 48 HPI on the three insect pest species of corn tested were estimated and summarized in Table 3. Interestingly, the lowest LC_{50} was observed in *S. litura* (8.89 IJ per larva), however, it was not significantly different compared with that observed in *O. furnacalis* (10.52 IJ per larva). Nevertheless, both were significantly lower as compared with the LC_{50} in *H. armigera* (19.98 IJ per larva). A different trend in virulence based on LC_{50} was observed for *S. abbasi* MBLB. The lowest LC_{50} was on *O. furnacalis* (10.98 IJ per larva) however, when compared with *S. litura* (17.08 IJ per larva), the difference was not significant. The highest LC_{50} for *S. abbasi* MBLB was on *H. armigera* (22.57 IJ per larva), which was significantly different from the LC_{50} of *O. furnacalis*, but not with the LC_{50} of *S. litura*. In addition, the LC_{50} of *H. indica* PBCB and *S. abbasi* MBLB revealed their equal level of virulence against *H. armigera* and *O. furnacalis*. In *S. litura*, however, *H. indica* PBCB was significantly more virulent than *S. abbasi* MBLB.

Lethal time assay

The median lethal time estimates (LT_{50}) of the *H. indica* PBCB and *S. abbasi* MBLB for the three lepidopteran pest species are presented in Table 4. Both *H. indica* PBCB and *S. abbasi* MBLB had significantly shortest LT_{50} of 21.90 and 24.18 hr, respectively, in *O. furnacalis*. In contrast, the significantly longest LT_{50} estimates of 30.96 and 32.64 hr were observed in *H. armigera* for *H. indica* PBCB and *S. abbasi* MBLB, respectively. Meanwhile, the LT_{50} values in *S. litura* of *H. indica* PBCB (24.31 hr) and *S. abbasi* MBLB (26.59 hr) were significantly different from the estimates in *H. armigera* and *O. furnacalis*. Results also showed that *H. indica* PBCB was significantly more virulent than *S. abbasi* MBLB against both *O. furnacalis* and *S. litura* but equally virulent to *H. armigera*.

Discussion

This study is the first most extensive research on EPN in the Philippines with molecular characterization for species identification and bioefficacy assays. The experiments demonstrated the pathogenicity of the local EPN isolates to some major lepidopteran pests in corn, and the identification of the two most virulent

Table 1. Species identification of the entomopathogenic nematode (EPN) populations in the Philippines based on molecular data.

Collection site (sample code)	Associated crop	Soil type/pH/% OM	Detected EPN species	% Nucleotide identity/ GenBank Acc. No. of significant hit	Assigned GenBank acc. nos. (for this paper)
Atisan, San Pablo, Laguna (ASPL3)	Bignay (<i>Antidesma bunius</i>)	Loam/6.1/4.40	<i>Steinernema minutum</i>	98/GU647156.1 (Maneesakorn et al., 2010)	KY807711
Curba, Calauan, Laguna (CCL4)	Cassava (<i>Manihot esculenta</i>)	Clay Loam/6.4/2.37	<i>S. minutum</i>	98/GU647156.1 (Maneesakorn et al., 2010)	KY807712
Mataas na Bayan, Lemery, Batangas (MBLB)	Sugarcane (<i>Saccharum officinarum</i>)	Sandy Loam/4.4/0.99	<i>Steinernema abbasi</i> (= <i>thermophilum</i>)	99/AY248749.1 (Nguyen and Byron, 2003)	KY807713
Puting Bato, Calaca, Batangas (PBCB)	Mango (<i>Mangifera indica</i>)	Sandy Loam/6.6/4.55	<i>Heterorhabditis indica</i>	100/GQ377411.1 (Khatri-Chhetri et al., 2010)	KY807714
Poblacion, Impasug-ong, Bukidnon (PIB2)	Sugarcane (<i>S. officinarum</i>)	Clay/5.2/3.79	<i>S. minutum</i>	98/GU647156.1 (Maneesakorn et al., 2010)	KY807715
Paraiso, Koronadal City, S. Cotabato (PKSC1)	Sugarcane (<i>S. officinarum</i>)	Sandy Loam/5.0/1.04	<i>Steinernema tami</i>	98/AY171280.1 (Spiridonov et al., 2004)	KY807716
San Rafael, Santo Tomas, Batangas (SRSTB3)	Corn (<i>Zea mays</i>)	Silty Clay Loam/5.0/2.43	<i>S. minutum</i>	98/GU647156.1 (Maneesakorn et al., 2010)	KY807717

OM, organic matter.

local strains, namely *H. indica* PBCB and *S. abbasi* MBLB, to *H. armigera*, *O. furnacalis*, and *S. litura*.

One of the limiting factors in EPN research in the Philippines is the unavailability of *Galleria mellonella*, the lepidopteran species conventionally used as host bait for EPN isolation from soil (Bedding and Akhurst, 1975), in the country. Although *G. mellonella* has been reported in the Philippines (Laigo and Morse, 1968), no laboratory or commercially reared colonies have been established in the country. Other than it is rarely found infesting apiaries since it commonly infests colonies of the less commonly commercially reared bee species, there is no market for this insect as fishing bait or feed

for pet animals in the country. Thus, the use of *O. furnacalis* as host bait was also explored basically because it is the most serious problem among the lepidopteran pests of corn, are easily reared in the laboratory, and has the closest phylogenetic relationship with *G. mellonella* (Regier et al., 2012). *Ostrinia furnacalis* belongs to the family Crambidae – a former subfamily of Pyralidae to where *G. mellonella* is still currently classified (Solis, 2007), while *H. armigera* and *S. litura* are both under the family Noctuidae.

In this study, we demonstrated the ability of *O. furnacalis* to recover EPN with varying virulence levels, suggesting that this insect species may serve

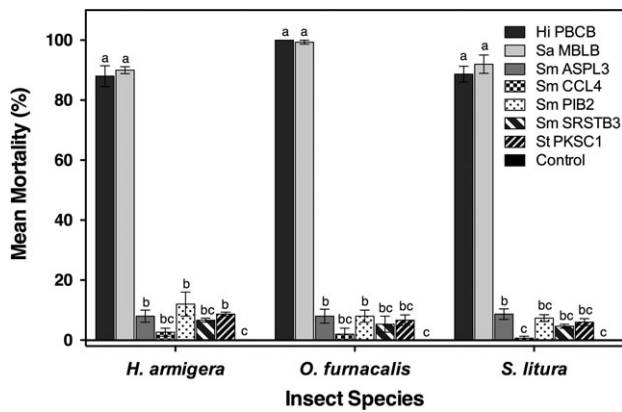


Figure 2: Mean percentage mortality of *Helicoverpa armigera*, *Ostrinia furnacalis*, and *Spodoptera litura* 48 hr post infection exposed to 200 IJ per larva of the Philippine entomopathogenic nematode isolates namely, *Heterorhabditis indica* PBCB (Hi PBCB), *Steinernema abbasi* MBLB (Sa MBLB), *Steinernema minutum* (Sm ASPL3, Sm CCL4, Sm PIB2 and Sm SRTSB3), and *Steinernema tami* PKSC1 (St PKSC1). Different letters for mean percentage mortality show significant difference between the EPN isolates within the insect species after two-way ANOVA followed by Tukey's multiple comparison test ($\alpha=0.05$).

as an alternative host bait. In addition, the use of the target insect as bait may provide a selective screening method for an increased probability of obtaining

EPN population highly virulent to insect species of interest as shown in this study. However, this protocol that offers a finer screening technique might not be as efficient as the *G. mellonella* baiting in studying EPN diversity due to underestimation of naturally-occurring EPN populations. Bedding and Akhurst (1975) accounted the very high susceptibility of *G. mellonella* larvae to EPN to their non-development of protective proteins since they are not naturally exposed to nematodes as compared with soil insects. In addition, Shapiro-Ilan et al. (2003) noted that utilization of the target insect pest as bait can be used to isolate EPN from soil that maybe host specific. Nonetheless, it will be interesting to compare the efficiency of *O. furnacalis* as a baiting host with *G. mellonella* as the former, having no developmental stage in the soil, is also not normally exposed to EPN. Moreover, a comparison of morphometric data of EPN cultured in *O. furnacalis* with those reared in *G. mellonella* is another noteworthy study to undertake.

Soil surveys covering different soil and habitat types (woodland, cropland, grassland) across Luzon, Visayas, and Mindanao areas revealed low positive detection of EPN (2.5% or 7 out of 279 soil samples). In a similar type of extensive sampling, low occurrence of EPN had also been reported in Azores, Portugal with 3.9% (46 out of 1,160 soil samples) (Rosa et al., 2000), in Indonesia with 11.7% (26 out of 223 soil samples) (Griffin et al., 2000), and in Ireland with 10.5% (58 out of 551 soil samples) detection (Griffin et al., 1991). However, a relatively higher EPN occurrence was observed in a soil sampling done in Liaoning (Northeast China), where there was 32.89% detection (49 out of 149 soil samples) from the samples (Wang et al., 2014). The

Table 2. Mean percentage penetration rate of *Heterorhabditis indica* PBCB and *Steinernema abbasi* MBLB in *Helicoverpa armigera*, *Ostrinia furnacalis*, and *Spodoptera litura* larvae at 48 hr post inoculation.

EPN isolate	Mean penetration rate (%) \pm SE		
	<i>H. armigera</i>	<i>O. furnacalis</i>	<i>S. litura</i>
Hi PBCB	14.35 \pm 0.49 ^a	14.25 \pm 0.35 ^a	18.18 \pm 1.35 ^a
Sa MBLB	28.15 \pm 2.51 ^b	14.54 \pm 0.41 ^a	23.15 \pm 0.29 ^b
Control	0.00 ^c	0.00 ^b	0.00 ^c

Different letters (a,b,c) for mean penetration rate show significant difference between the EPN isolate within the insect species after two-way ANOVA and Tukey's multiple comparison test ($\alpha = 0.05$). SE, standard error.

Table 3. Median lethal concentration (LC_{50}) estimates of the Philippine *Heterorhabditis indica* PBCB (Hi PBCB) and *Steinernema abbasi* MBLB (Sa MBLB) isolates to lepidopterous pests of corn, *Helicoverpa armigera*, *Ostrinia furnacalis*, *Spodoptera litura*.

EPN isolate	Insect species	LC_{50} (IJs/larva)	95% Fiducial limit		Slope \pm SE	χ^2	df
			Lower	Upper			
Hi PBCB	<i>H. armigera</i>	19.98 ^b	16.63	24.32	1.58 \pm 0.16	1.87	3
	<i>O. furnacalis</i>	10.52 ^a	8.82	12.74	1.65 \pm 0.16	1.65	3
	<i>S. litura</i>	8.89 ^a	6.98	10.98	2.857 \pm 0.24	3.46	3
Sa MBLB	<i>H. armigera</i>	22.57 ^b	18.80	27.61	1.60 \pm 0.16	0.98	3
	<i>O. furnacalis</i>	10.98 ^a	8.25	15.21	1.88 \pm 0.17	3.60	3
	<i>S. litura</i>	17.08 ^{ab}	12.27	24.07	2.03 \pm 0.17	3.77	3

SE, standard error; χ^2 , Pearson χ^2 ; df, degrees of freedom. a, b, ab; LC_{50} values within the EPN isolate followed by the same letter were not significantly different due to overlapping 95% fiducial limit values.

higher percentage of occurrence of *Steinernema* species observed in this study is consistent to all these aforementioned related researches as well as studies in Europe (Hominick et al., 1995). Nyasani et al. (2008) noted that *Steinernema* spp. are more widespread than *Heterorhabditis* since the former

has a greater ability to adapt to their environment than the latter.

In similar studies on the use of EPN against lepidopteran pests, Kalia et al. (2014) proved the efficacy of *S. thermophilum* (= *abbasi*) against *H. armigera* and *S. litura*. With regards to host susceptibility of

Table 4. Median lethal time (LT_{50}) estimates of Philippine *Heterorhabditis indica* PBCB (HiPBCB) and *Steinernema abbasi* MBLB (SaMBLB) isolates on lepidopterous pests of corn *Helicoverpa armigera*, *Ostrinia furnacalis*, *Spodoptera litura*. Lethal times were estimated using 200 IJ/larvae within 48 hr post infection.

EPN isolate	Insect species	LT_{50} (h)	95% Fiducial limit		Slope \pm SE	χ^2	df
			Lower	Upper			
Hi PBCB	<i>H. armigera</i>	30.962 ^c	29.835	32.015	6.947 \pm 0.954	0.224	5
	<i>O. furnacalis</i>	21.901 ^a	21.273	22.440	12.797 \pm 1.059	3.684	5
	<i>S. litura</i>	24.309 ^b	23.567	24.994	8.763 \pm 0.826	0.802	5
Sa MBLB	<i>H. armigera</i>	32.264 ^c	29.929	35.835	3.892 \pm 0.514	1.423	4
	<i>O. furnacalis</i>	24.177 ^a	23.322	24.960	10.282 \pm 0.882	0.253	3
	<i>S. litura</i>	26.586 ^b	25.847	27.312	12.036 \pm 0.938	2.119	3

SE, standard error; χ^2 , Pearson χ^2 ; df, degrees of freedom.

the different lepidopteran species to *S. abbasi*, they reported that the LC_{50} at 36 hr after treatment for *S. litura* and *H. armigera* was 85 and 54.68 IJ per larva, respectively. In comparison with the result of our study, the LC_{50} at 48 HPI of the local *S. abbasi* MBLB isolate for both test insects was relatively lower with values of 22.57 IJ per larva for *H. armigera* and 17.08 IJ per larva for *S. litura*. The variation between the studies can be attributed to the difference on the larval instars used, last instar for the former and third instars for this study, and probably the longer incubation time. However, the possibility of a higher efficacy or phenotypic fitness for the Philippine *S. abbasi* MBLB can also be a substantial reason for such intraspecific variation. Earlier studies had already implicated significant differences in the efficacy of various nematode species or strains for controlling a particular insect (Bedding et al., 1983) as the biological control potential of a particular EPN is greatly influenced by the penetration rate of IJs, the time of symbiotic bacteria discharge, and the virulence of these symbionts (Glazer and Navon, 1990). In a more recent study, the intraspecific variability of local strains of *S. feltiae* in Spain in terms of reproduction, virulence, and the physiological and molecular profile of the endosymbiont was demonstrated (Campos-Herrera and Gutierrez, 2014).

When the virulence of the two local EPN was compared within the insect species, a significant difference in the LC_{50} values was only observed in *S. litura*, in which *H. indica* PBCB was more virulent than *S. abbasi* MBLB. *H. indica* PBCB and *S. abbasi* MBLB exhibited the same degree of virulence in both *H. armigera* and *O. furnacalis*. Among the three test insects, *S. litura* has the softest cuticle, which probably made its intersegmental membranes more vulnerable to abrasions caused by *H. indica* using its dorsal tooth.

Significant difference in the LT_{50} values was observed in the three lepidopteran species infected with *H. indica* PBCB and *S. abbasi* MBLB. Based on LT_{50} estimates for both EPN, the ranking of the susceptibility of the test insects from the most susceptible to the least susceptible is as follows: *O. furnacalis* > *S. litura* > *H. armigera*. Significant difference in the LT_{50} values was observed among the three test insects infected with *H. indica* PBCB. The same trend was observed with those infected with *S. abbasi* MBLB. The innate susceptibility of *O. furnacalis* to both EPN can be attributed to the long-term dependence of these isolates to the insect as food source. From the time of soil-baiting to mass production, the EPN have been conditioned to consume *O. furnacalis* in the absence of *G. mellonella*.

Consistently, *H. armigera* infected with both EPN species displayed the highest LC_{50} and LT_{50} values for both EPN species indicative of its relative resilience against EPN parasitism. Aside from host preference and nematode fitness stated earlier, the activation of insect's immune response, be it cuticular, humoral, cellular, or combinations, to different invading pathogens including EPNs might have played a great role in this interaction (Castillo et al., 2011; Krautz et al., 2014).

The local *S. minutum* and *S. tami* were found to be pathogenic to the three lepidopteran species tested, however, they exhibited very low virulence. Although they were able to complete their life cycle in the test insects, their ability to efficiently kill the host was not evident. These EPN could have other suitable insect species as hosts.

Surveys coupled with proper identification of EPN, and comprehensive bioassays, product formulation, and field-efficacy assessment are fundamental requirements in the employment of a viable EPN technology-based program. As EPN study in the Philippines is still in its infancy, this baseline information on the diversity of these nematodes and their occurrence across different geographical locations; and their biological control potential against lepidopteran insect pests of corn contributes to the growing basic and applied local EPN researches and provides a promising insect pest management option that answers to both food security and safety in the country. Further, the development of an indigenous EPN-based technology could lead to a formulation of an effective inoculative and/or inundative biological control strategy, which can also be incorporated in integrated pest management of local insect pests. However, whether these local EPN are more or less virulent to the lepidopterous pests tested as compared with exotic strains or even the commercial strains needs further examination, since foraging strategy in combination with the target insect pest as well as ecological cues are among the important factors in EPN bioefficacy (Berry et al., 1997; Shapiro-Ilan and Cottrell, 2005).

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